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Therapeutic effects of lysophosphatidylcholine in experimental sepsis

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Sepsis represents a major cause of death in intensive care units. Here we show that administration of lysophosphatidylcholine (LPC), an endogenous lysophospholipid, protected mice against lethality after cecal ligation and puncture (CLP) or intraperitoneal injection of *Escherichia coli*. *In vivo* treatment with LPC markedly enhanced clearance of intraperitoneal bacteria and blocked CLP-induced deactivation of neutrophils. *In vitro*, LPC increased bactericidal activity of neutrophils, but not macrophages, by enhancing H_2O_2 production in neutrophils that ingested *E. coli*. Incubation with an antibody to the LPC receptor, G2A, inhibited LPC-induced protection from CLP lethality and inhibited the effects of LPC in neutrophils. G2A-specific antibody also blocked the inhibitory effects of LPC on certain actions of lipopolysaccharides (LPS), including lethality and the release of tumor necrosis factor-α (TNF-α) from neutrophils. These results suggest that LPC can effectively prevent and treat sepsis and microbial infections.

Sepsis is a major cause of death in intensive care units, accounting for over 200,000 deaths per year in the United States alone[1]. Increasing evidence suggests that sepsis impairs immune function by inducing defects in innate immunity[2–4] and excessive lymphocyte apoptosis[5–7]. The resulting immunosuppression has been suggested to be a major contributing factor in sepsis-induced mortality[4,8,9]. Accordingly, activation of macrophages with interferon-γ (IFN-γ) in septic patients[4], blockade of complement-induced neutrophil dysfunction[3,10] and inhibition of lymphocyte apoptosis[11] in animals with experimentally induced sepsis have all been reported to have beneficial effects.

LPC is a major component of oxidized low-density lipoprotein. LPC has various stimulatory effects in many types of immune cells *in vitro* (reviewed in ref. 12), including monocytes[13–16], macrophages[17–21], T lymphocytes[22–26] and neutrophils[27–29]. We therefore hypothesized that LPC could be useful for treating acute episodes of immune disruption that occur in the context of sepsis. We have investigated the effects of LPC treatment on three mouse models of sepsis, including CLP, which closely mimics human acute peritonitis and is regarded as the most clinically relevant animal model of sepsis.

RESULTS

LPC protects against sepsis-induced lethality

CLP was performed on albino ICR (Institute of Cancer Research) mice, as described in Methods. Animals were subcutaneously (s.c.) injected with various doses of 18:0 (one-position fatty acid chain length/degree of saturation) LPC or with vehicle (PBS containing 2% BSA). Injections were given four times at different adjacent sites, at 12-h intervals beginning 2 h after CLP. Mice were then observed for up to 10 d after CLP. 18:0 LPC provided significant protection against CLP-induced lethality at a dose of 1 mg/kg ($P < 0.01$), with higher levels of protection afforded at 10 and 20 mg/kg (**Fig. 1a**). We then varied the number of 18:0 LPC injections given after CLP from one to five, with injections given every 12 h starting 2 h after CLP. A significant improvement in the long-term survival rate was observed when animals were given a minimum of four injections of 18:0 LPC (10 mg/kg; $P < 0.05$; **Fig. 1b**). These findings suggest that treatment with 18:0 LPC should be maintained for at least 48 h after CLP to significantly increase the long-term survival rate (**Supplementary Note** online). Plasma LPC in intact mice injected with 18:0 LPC (10 mg/kg, s.c.) was increased by 11% 1 h after injection (from 577 ± 17 to 633 ± 17 μM; $P < 0.05$), but LPC levels returned to normal (571 ± 13 μM) within 4 h after injection. The injection site showed a slight increase in infiltration of inflammatory cells when examined 24 h after injection.

We next asked whether delayed 18:0 LPC treatment could still protect mice against CLP-induced lethality. We treated mice with four 10 mg/kg (s.c.) injections of 18:0 LPC at 12-h intervals, beginning 10 h after CLP. Even when the initiation of treatment was delayed, 18:0 LPC retained nearly its full protective effect against CLP-induced lethality ($P < 0.001$; **Fig. 1c**). The protective effect was largely lost, however, when LPC treatment was delayed until 16 h after CLP (data not shown and **Supplementary Note** online).

To determine whether different molecular species of LPC differ in their activities, LPC molecules with acyl chains of varying lengths were administered four times at doses equimolar to 10 mg/kg of 18:0

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LPC, at 12-h intervals beginning 2 h after CLP (Fig. 1d). 18:0 LPC and 18:1 LPC were the most effective ($P < 0.001$), suggesting that saturation of the acyl chain may not be required. 16:0 LPC had no significant effect on the overall 10-d survival rate, whereas 14:0 LPC modestly improved it ($P < 0.05$). Other lysophospholipids, including lysophosphatidylserine, lysophosphatidylethanolamine, lysophosphatidylinositol, lyso-platelet activating factor and sphingosylphosphorylcholine, did not significantly improve the survival rate when administered at the same dose, although lysophosphatidate did improve survival (data not shown). In contrast, platelet-activating factor (PAF) increased CLP-induced lethality (data not shown). We also examined whether 18:0 LPC treatment protects mice against sepsis induced by direct intraperitoneal (i.p.) injection of live *E. coli*. The mortality induced by i.p. injection of 10^8 live *E. coli* cells was effectively inhibited ($P < 0.05$) by 18:0 LPC treatment (10 mg/kg, s.c., four times at 12-h intervals beginning 2 h after *E. coli* injection; Fig. 1e).

We next examined the role of G2A, which was recently identified as a receptor for LPC[30], in the protective effects of LPC. Mice were intravenously pretreated with G2A-specific antibody (80 μg/mouse) 24 h before CLP. Control mice received normal goat IgG (80 μg/mouse). Pretreatment with G2A-specific antibody blocked the protective effects of LPC (10 mg/kg, s.c., four times at 12-h intervals beginning 2 h after CLP) against CLP-induced lethality ($P < 0.01$; Fig. 1f).

Effects of LPC on CLP-induced cytokine levels

The effects of 18:0 LPC on CLP-induced cytokine levels in peritoneal lavage fluid were examined from 4 h to 3 d after CLP (Fig. 2). At early time points, 18:0 LPC treatment induced small and transient changes in the levels of certain CLP-induced cytokines. TNF-α and interleukin-1β (IL-1β) were decreased (Fig. 2a,b), whereas T-helper type 1 cytokines (IFN-γ, IL-2 and IL-12p40) were increased (Fig. 2c,d,g). T-helper type 2 cytokines (IL-6 and IL-10) were not affected by treatment with 18:0 LPC (Fig. 2e,f).

LPC enhances bacterial clearance *in vivo*

We next examined whether LPC treatment enhances clearance of CLP-induced intraperitoneal bacteria. In mice treated with 18:0 LPC (10 mg/kg, s.c., 2 h and 14 h after CLP), intraperitoneal bacterial counts were decreased by 80% 24 h after CLP (Fig. 3a). Similarly, 18:0 LPC treatment (10 mg/kg, s.c., 2 h and 14 h after *E. coli* injection) enhanced bacterial clearance in mice that were injected intraperitoneally with 10^8 live *E. coli* cells (Fig. 3a). However, 18:0 LPC had no direct bactericidal effect when it was incubated 24 h *in vitro* with *E. coli* at concentrations up to 100 μM (data not shown).

LPC blocks CLP-induced neutrophil deactivation

Neutrophils derived from mice subjected to CLP are impaired in their ability to produce H_2O_2 *in vitro*, suggesting that CLP deactivates neutrophils[3]. To determine whether LPC treatment prevents CLP-induced deactivation of neutrophils, blood neutrophils were isolated from mice 24 h after CLP. Neutrophils were then stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) *in vitro*, and H_2O_2 production was assayed. Treatment with 18:0 LPC (10 mg/kg, s.c., at 2 h and 14 h after CLP) effectively blocked CLP-induced impairment of neutrophil H_2O_2 production (Fig. 3b).

LPC increases bactericidal activity of neutrophils

We investigated whether LPC increases bactericidal activity when applied directly to phagocytes isolated from intact mice. Neutrophils were allowed to ingest *E. coli* for 1 h, and the extracellular *E. coli* were washed out. Neutrophils were then exposed to 30 μM 18:0 LPC for 15 min, and the number of viable intracellular *E. coli* cells was counted. Treatment with 18:0 LPC (30 μM) markedly

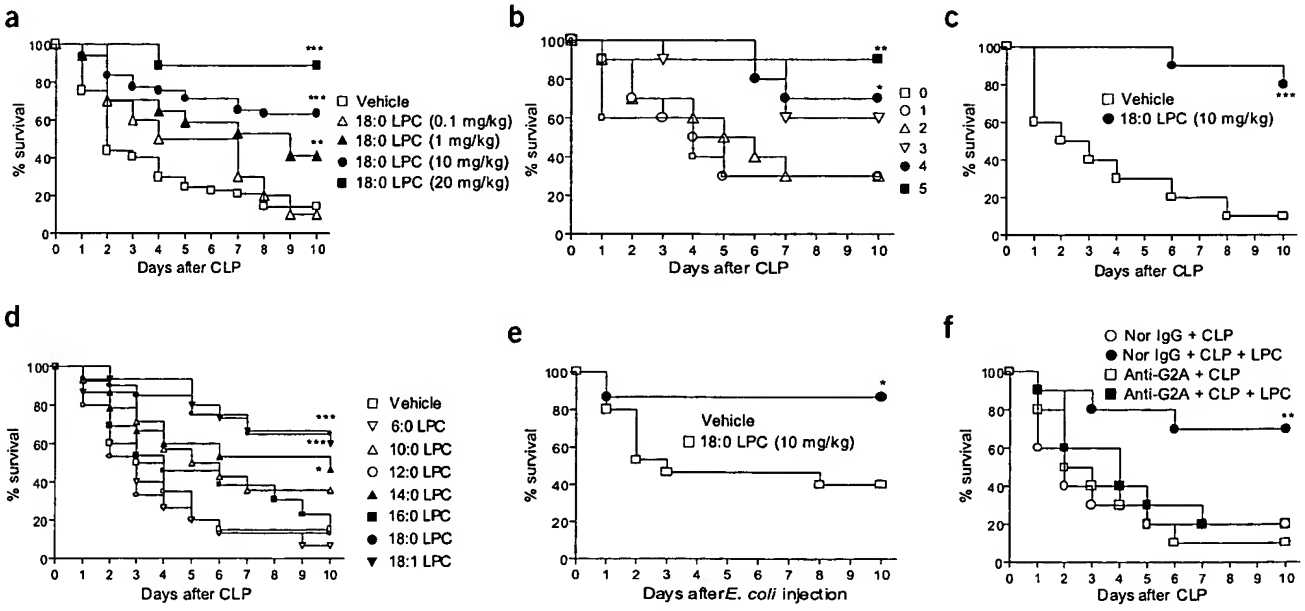

Figure 1 LPC protects against sepsis-induced lethality. (**a**) CLP mice were administered various doses of 18:0 LPC. (**b**) CLP mice were given 0, 1, 2, 3, 4 or 5 injections of 18:0 LPC (10 mg/kg). (**c**) Delayed treatment with 18:0 LPC, beginning 10 h after CLP, still protected mice against CLP-induced lethality. (**d**) Effect of various molecular species of LPC on survival. (**e**) 18:0 LPC protects against lethality induced by i.p. injection of *E. coli*. (**f**) Pretreatment with G2A-specific antibody (anti-G2A) inhibits the protective effects of 18:0 LPC against CLP-induced lethality. Nor IgG, normal goat IgG. *, $P < 0.05$; **, $P < 0.01$; ***; $P < 0.001$ compared with vehicle (**a–e**) or anti-G2A-pretreated group (**f**). $n = 10–16$ mice per group.

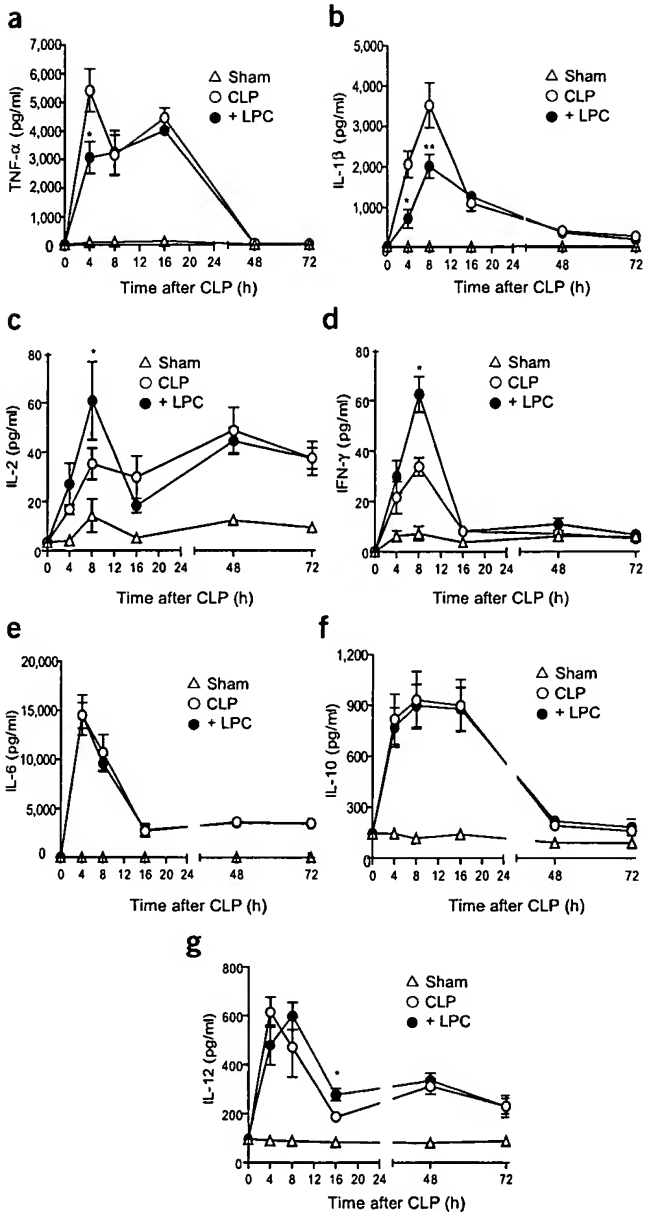

Figure 2 Effects of 18:0 LPC on various cytokines in peritoneal lavage fluid of CLP-treated mice. (**a–g**) Separate groups of animals were subjected to either sham CLP, CLP alone or CLP plus LPC treatment (+ LPC). **a**, TNF-α; **b**, IL-1β; **c**, IL-2; **d**, IFN-γ; **e**,IL-6; **f**, IL-10; **g**, IL-12. Data are expressed as mean ± s.e.m. ($n = 8$). *, $P < 0.05$; **, $P < 0.01$ compared with CLP alone.

increased bactericidal activity of neutrophils (Fig. 4a). In contrast, 16:0 LPC, lysophosphatidylserine and lysophosphatidylethanolamine at 30 μM had no effect (data not shown). Unlike neutrophils, the bactericidal activity of macrophages was not increased in response to 18:0 LPC (data not shown). The LPC-induced increase in neutrophil bactericidal activity was inhibited by G2A-specific antibody (Fig. 4a). In addition, G2A mRNA was detected in neutrophils by RT-PCR (Fig. 4b).

Because H_2O_2 production has a crucial role in the bactericidal activity of phagocytes, we examined H_2O_2 production under the same experimental conditions (Fig. 4c) as in the bactericidal activity assay (Fig. 4a). Treatment of neutrophils with 18:0 LPC (30 μM) induced a significant increase (39%) in H_2O_2 production. Notably, 18:0 LPC induced a marked enhancement (92%) in H_2O_2 production specifically in neutrophils that had ingested *E. coli*. Ingestion of the same amount of *E. coli* without LPC did not induce H_2O_2 production in neutrophils. 18:0 LPC (30 μM) also induced a large increase in H_2O_2 production in macrophages. Unlike neutrophils, however, 18:0 LPC did not further enhance H_2O_2 production in macrophages that had ingested *E. coli*, and *E. coli* ingestion alone markedly increased H_2O_2 production in macrophages.

Incubation with G2A-specific antibody (1 μg/ml) effectively inhibited the 18:0 LPC–induced increase in H_2O_2 production in neutrophils (Fig. 4d). We examined the effects of various species of LPC on H_2O_2 production in blood neutrophils (Fig. 4e) and found that the rank order of efficacy of the various LPCs in neutrophils paralleled the survival data (Fig. 1e). That is, 18:0 LPC, 18:1 LPC and, to a lesser extent, 14:0 LPC were most effective in neutrophils, whereas 18:0 LPC and 18:1 LPC were effective in macrophages (Fig. 4e). At a concentration of 30 μM, lysophosphatidate induced H_2O_2 production in neutrophils, but lysophosphatidylserine, lysophosphatidylethanolamine, lysophosphatidylinositol and sphingosylphosphorylcholine did not (data not shown).

LPC inhibits effects of LPS

We examined the effects of LPC on sepsis induced by LPS, a major causative agent of sepsis. The mortality induced by an i.p. injection of LPS (5 mg/kg) was effectively inhibited when 18:0 LPC (10 mg/kg, s.c.) was administered four times at 12-h intervals beginning 2 h after LPS injection ($P < 0.05$; Fig. 5a). The same dosage of lysophosphatidylethanolamine, lysophosphatidylinositol, lyso-platelet activating factor and sphingosylphosphorylcholine had no effect on mortality (data not shown). 18:0 LPC treatment (10 mg/kg, s.c.) affected plasma TNF-α, IL-1β, and IFN-γ levels in the same manner as in CLP-treated mice (Fig. 5b): the increase in plasma TNF-α and IL-1β was significantly attenuated 1.5 h after LPS injection ($P < 0.05$), and the increase in plasma IFN-γ was markedly enhanced 6 h after LPS injection ($P < 0.001$).

We next examined the effects of 18:0 LPC on basal and LPS-induced release of TNF-α and IL-1β from neutrophils and macrophages. 18:0 LPC effectively inhibited basal and LPS-induced TNF-α release from neutrophils, but not from macrophages (Fig. 5c). LPC at 30 μM significantly ($P < 0.01$) increased IL-1β release from macrophages (Fig. 5d). This is consistent with the previous report of LPC-induced increase in IL-1β production from monocytes[15]. To examine the involvement of G2A in LPC protection against LPS-induced lethality, mice were intravenously pretreated with G2A-specific antibody (80 μg/mouse) 24 h before LPS injection. Control mice received equivalent injections of normal goat IgG (80 μg/mouse). The antibody pretreatment blocked the protective effects of LPC (10 mg/kg, s.c., four times at 12-h intervals beginning 2 h after LPS injection) against LPS-induced lethality ($P < 0.05$). Mice that received normal goat IgG, LPS and 18:0 LPC had a 100% (10/10) survival rate (percentage of mice that survived 10 d after LPS injection), whereas mice that received G2A-specific antibody, LPS and 18:0 LPC had a 60% (6/10) survival rate, and mice that received normal goat IgG, LPS and G2A-specific antibody had a 50% (5/10) survival rate. Pretreatment with G2A-specific antibody *in vitro* blocked the LPC-mediated inhibition of LPS-induced TNF-α release from neutrophils (Fig. 5e).

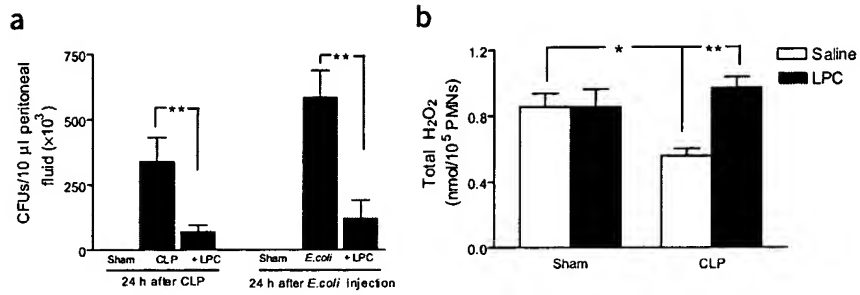

Figure 3 LPC enhances bacterial clearance *in vivo* and blocks CLP-induced deactivation of neutrophils. (a) Bacterial count (CFUs) in peritoneal lavage fluid, 24 h after CLP or i.p. injection of 10^8 live *E. coli* cells. + LPC, CLP plus LPC treatment. (b) PMA-induced H_2O_2 production from neutrophils (PMNs) isolated 24 h after CLP. 18:0 LPC (10 mg/kg, s.c.) was administered at 2 h and 14 h after sepsis induction. Data are expressed as mean ± s.e.m. (a, $n = 16$; b, $n = 9$). *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

We found that therapeutic administration of LPC after the induction of sepsis effectively inhibited lethality induced by three mouse models of sepsis. Particularly in CLP, the most reliable animal model for human sepsis, LPC was a potent and efficacious antiseptic agent, with a minimal effective dose of 1 mg per kg per 12 h, and a wide therapeutic window (effective up to as late as 10–24 h after CLP, depending on the severity of the CLP model). LPC exerted at least two distinct antiseptic mechanisms: it enhanced bacterial elimination and inhibited the actions of LPS. This dual mechanism of action makes LPC ideally suited for treating sepsis.

Defects in innate immunity, particularly dysfunctions of neutrophils[3,31,32] and monocytes[4,31], are believed to contribute to sepsis-induced mortality. LPC markedly enhanced the *in vivo* elimination of bacteria introduced by CLP or by *E. coli* injection. LPC directly increased the *in vitro* bactericidal activity of neutrophils; this effect was closely correlated with enhanced H_2O_2 production in neutrophils that had ingested *E. coli*. In contrast, bactericidal activity and H_2O_2 production were not further enhanced by LPC in macrophages that had ingested *E. coli*. Moreover, the molecular species of LPC that had effects on H_2O_2 production in neutrophils (Fig. 4e) closely matched the ones effective on CLP-induced lethality (Fig. 1d). The CLP-induced deactivation of neutrophils was blocked by LPC treatment. Taken together, these findings suggest that neutrophils, rather than macrophages, are the principal target cells that mediate the LPC-induced enhancement of bacterial elimination. The finding that LPC enhances neutrophil bactericidal activity has important implications, because invading microbes are predominantly cleared by neutrophils, the major phagocytes within the circulation. However, the possibility that monocytes and/or macrophages contribute, at least in part, to the enhanced bacterial elimination cannot be excluded, as LPC has many stimulatory effects on monocytes and macrophages[13–21].

G2A and GPR4 have recently been identified as LPC receptors[30,33]. In this study, we showed that G2A mRNA is expressed in neutrophils, and that a G2A-specific antibody effectively inhibits the LPC-induced increase in bactericidal activity and H_2O_2 production in neutrophils. Our findings also indicate that G2A has a crucial role in the protective effects of LPC against CLP- and LPS-induced lethality. Sphingosylphosphorylcholine, a less potent G2A agonist[30], increased the 10-d survival rate of CLP mice at a dose higher than that used for LPC (30 mg/kg, s.c., four times at 12-h intervals beginning 2 h after CLP; data not shown). This further supports the involvement of G2A in LPC protection against sepsis. 18:0 LPC, 18:1 LPC and 16:0 LPC, but not 14:0 LPC, were identified as effective ligands of G2A[30]. Thus, the lack of efficacy of 16:0 LPC and the modest efficacy of 14:0 LPC (Figs. 1d and 4e) are not completely consistent with the reported

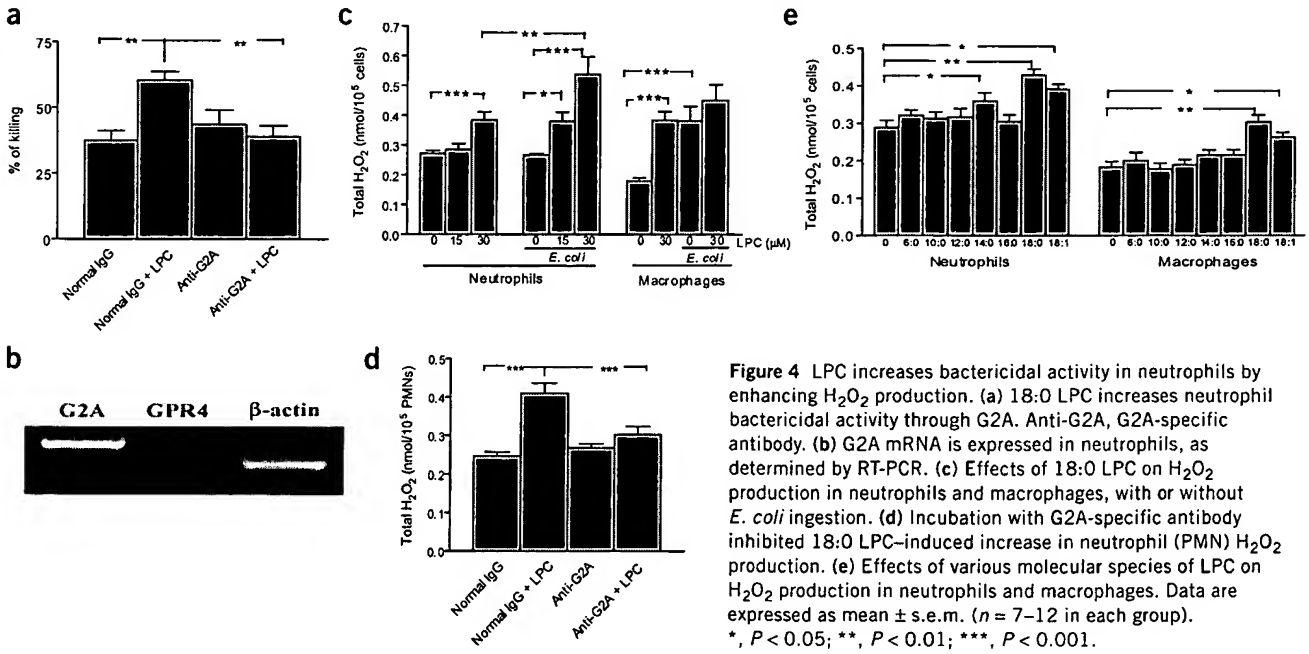

Figure 4 LPC increases bactericidal activity in neutrophils by enhancing H_2O_2 production. (a) 18:0 LPC increases neutrophil bactericidal activity through G2A. Anti-G2A, G2A-specific antibody. (b) G2A mRNA is expressed in neutrophils, as determined by RT-PCR. (c) Effects of 18:0 LPC on H_2O_2 production in neutrophils and macrophages, with or without *E. coli* ingestion. (d) Incubation with G2A-specific antibody inhibited 18:0 LPC–induced increase in neutrophil (PMN) H_2O_2 production. (e) Effects of various molecular species of LPC on H_2O_2 production in neutrophils and macrophages. Data are expressed as mean ± s.e.m. ($n = 7$–12 in each group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

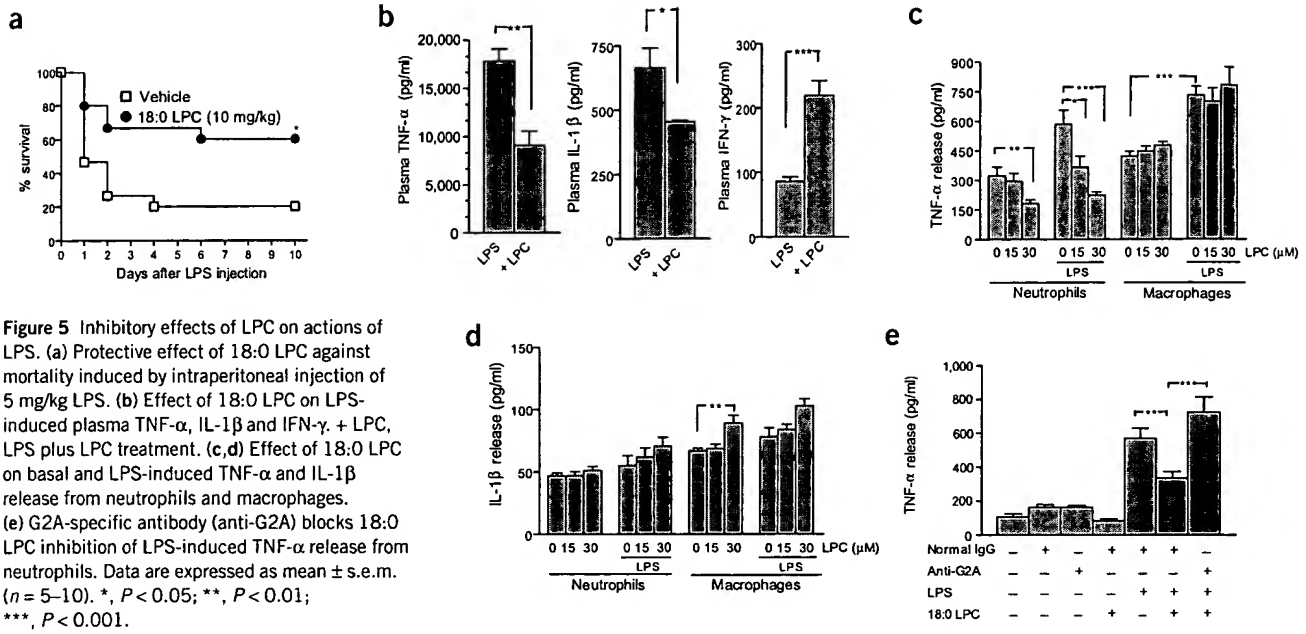

Figure 5 Inhibitory effects of LPC on actions of LPS. (**a**) Protective effect of 18:0 LPC against mortality induced by intraperitoneal injection of 5 mg/kg LPS. (**b**) Effect of 18:0 LPC on LPS-induced plasma TNF-α, IL-1β and IFN-γ. + LPC, LPS plus LPC treatment. (**c**,**d**) Effect of 18:0 LPC on basal and LPS-induced TNF-α and IL-1β release from neutrophils and macrophages. (**e**) G2A-specific antibody (anti-G2A) blocks 18:0 LPC inhibition of LPS-induced TNF-α release from neutrophils. Data are expressed as mean ± s.e.m. ($n = 5$–10). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ligand-binding profile of G2A[30]. In neutrophils and monocytes, G2A may prefer 18:0 LPC over 16:0 LPC. The significantly lower activity of 16:0 LPC, as compared with 18:0 or 18:1 LPC has been reported in neutrophils[29,34] and monocytes[14].

LPC induced a modest and transient change in the levels of certain cytokines at very early stages in the CLP model. T-helper type 1 cytokines (IFN-γ, IL-2 and IL-12) were increased in response to LPC, whereas the proinflammatory cytokines TNF-α and IL-1β were decreased. A very similar pattern of cytokine regulation was observed in the LPC-treated LPS model (Fig. 5b). These differential effects of LPC on different cytokines may result from LPC acting on distinct sets of target cells. Increases in IFN-γ[4,35], IL-2 (ref. 36) and IL-12 (refs. 37,38), as well as the combined decrease of TNF-α and IL-1β[39], have been reported to have beneficial effects in sepsis. Because of the modest and transient nature of these effects, these LPC-induced changes in cytokine levels may not have a major role in the LPC-induced increase in survival. We cannot, however, exclude the possibility that the combined effect of all these changes in cytokines may, at least in part, contribute to improved survival. In future studies, it will be interesting to examine the effect of combining supplementation with IFN-γ, IL-2 and IL-12 and blockade of TNF-α and IL-1β in sepsis.

We also showed that 18:0 LPC inhibited LPS activity in this study. 18:0 LPC effectively inhibited LPS-induced lethality and the release of TNF-α from neutrophils in response to LPS. Both of these effects were efficiently blocked by G2A-specific antibody. Neutrophils have been proposed to be the major cell type that produces TNF-α in response to LPS challenge in mice[40]. Thus, the LPC-induced inhibition of very early–phase plasma TNF-α in the LPS (Fig. 5b) and CLP (Fig. 2a) models may have been caused by the actions of LPC on neutrophils, rather than on monocytes and macrophages. The 18:0 LPC–induced inhibition of LPS may not be confined to neutrophils, as 18:0 LPC inhibited both basal and LPS (1 μg/ml)–induced IL-8 release from human peripheral blood monocytes (data not shown).

It was recently reported that plasma LPC is significantly decreased in septic patients, and that patients who die of sepsis have significantly lower plasma LPC than patients who survive a septic episode[41]. These clinical findings support our hypothesis that supplementation with LPC may be beneficial for patients with sepsis. LPC is one of the metabolites derived from the oxidation of low-density lipoprotein, and these metabolites are thought to be involved in the pathogenesis of atherosclerosis[42]. However, the beneficial effects of treating sepsis with LPC in the short term (possibly within a week) could far exceed the potential atherogenic effects of this lipid, as LPC could prevent the devastating consequences of sepsis. Appropriate caution should be used in patients with cardiac ischemia, however, because 16:0 LPC may cause electrophysiological alterations in ischemic myocardium[43]. At the doses used in this study, LPC did not induce any apparent toxic effects in mice (data not shown). In addition to sepsis, the enhancing effect of LPC on neutrophil bactericidal activity should be useful in cases of microbial infections that have not yet progressed to sepsis. This new approach for combating microbial infections would be complementary to the approach of directly attacking microbial pathogens with antimicrobial agents. Such an approach could be important, considering the continuous appearance of new pathogenic microbes that are resistant to the currently available antimicrobial agents. In conclusion, we have identified a new therapeutic application of LPC for use in sepsis and microbial infections. These findings suggest that a clinical evaluation of these effects of LPC will be useful.

METHODS

Animals and sepsis models. We used male ICR mice and, in a subset of experiments, male C57BL/6 and female BALB/c mice. Procedures for animal experiments were approved by the Animal Experimentation Committee at Hallym University. For CLP, mice were anesthetized with pentobarbital (50 mg/kg, i.p.), a small abdominal midline incision was made, and the cecum was exposed. The cecum was mobilized and ligated below the ileocecal valve, punctured through both surfaces twice or once (for less severe CLP) with a 22-gauge needle, and the abdomen was closed. Mice subjected to sham CLP underwent the same procedure, except for ligation and puncture of the cecum. For other sepsis models, mice were injected i.p. with either a bacterial suspension containing 10^8 live *E. coli* cells (DH5-α, Invitrogen) or LPS (5 mg/kg; *E. coli* O55:B5, Sigma). Survival was monitored once daily for 10 d.

Measurement of cytokine and LPC levels. For measurement of CLP-induced cytokines in peritoneal lavage fluids, mice were given 18:0 LPC at 2 h, 16 h, 28 h and 40 h after CLP. Peritoneal lavage fluid (~2 ml recovered from each mouse) was collected at various times between 4 h and 72 h after CLP. For measurement of LPS-induced plasma cytokines, mice were given 18:0 LPC 30 min after injection of LPS, and plasma was collected 1 h (for TNF-α and IL-1β) or 5.5 h (for IFN-γ) later. Concentrations of cytokines were measured with an enzyme-linked immunoassay kit (Biosource International). Plasma LPC concentrations were assayed as described previously[44], based on the standard curve for 18:0 LPC.

Determination of colony-forming units (CFUs). Twenty-four hours after CLP, mice were anesthetized and peritoneal lavage fluid was collected. The peritoneal lavage fluid was cultured overnight on blood-agar base plates (Trypticase Soy Agar Deeps, Becton Dickinson) at 37 °C, and the CFUs were counted.

Isolation of neutrophils and macrophages. Neutrophils were isolated from peripheral blood using a discontinuous Percoll gradient (1.072 and 1.089) as previously described[45]. Macrophages were obtained from peritoneal exudate cells by peritoneal lavage with 2 ml of cold PBS.

Measurement of H_2O_2. Neutrophils isolated from CLP mice were stimulated with PMA (100 ng/ml; Sigma) for 1 h (Fig. 3b). Blood neutrophils and peritoneal macrophages in fresh phenol red–free RPMI 1640 (supplemented with 5% FBS) were incubated with various LPCs at a concentration of 30 μM for 2 h (Fig. 4e). In some experiments, blood neutrophils were preincubated with either G2A-specific antibody (1 μg/ml) or normal goat IgG (1 μg/ml) for 0.5 or 1 h. 18:0 LPC was then added to the medium at a final concentration of 30 μM, and H_2O_2 production was assayed 2 h after the addition of LPC. H_2O_2 was measured in the supernatants with an H_2O_2 assay kit (Oxis International). The G2A-specific antibody (M-20, Santa Cruz) and normal goat IgG were dialyzed overnight in PBS before use.

Neutrophil bactericidal activity. Neutrophils were incubated at 37 °C on 13-mm plastic cover slips in 60-mm plastic culture dishes (10^6 neutrophils per cover slip; 6–8 cover slips per dish) for 1 h, and nonadherent cells were removed. Neutrophils were then incubated with 10^6 opsonized *E. coli* cells for 1 h. After washing out unengulfed *E. coli*, the number of viable bacteria in neutrophils was determined before and after further incubation with 30 μM 18:0 LPC or vehicle for 1 h. The percentage of bacteria killed was calculated as 100 × (1 – CFUs after LPC exposure/CFUs before LPC exposure)[46]. The supernatants were collected to measure H_2O_2 production (Fig. 4c). For experiments with G2A-specific antibody, blood neutrophils were incubated with either G2A-specific antibody (1 μg/ml) or normal goat IgG (1 μg/ml) during exposure to *E. coli* for 1 h, and during the subsequent exposure to LPC for 1 h (Supplementary Methods online).

RT-PCR. Total cellular RNA was extracted from mouse blood neutrophils. Primer sequences were as follows[33,47]: β-actin, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ (forward) and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (reverse); mouse GPR4, 5′-CTACCTGGCTGTGGCTCAT-3′ (forward) and 5′-CAAAGACGCGGTATAGATTCA-3′ (reverse); mouse G2A, 5′-CAGGACTGGCTTGGGTCATT-3′ (forward) and 5′-TAGCGGTCGCAGGAAATGCAG-3′ (reverse). The PCR products were electrophoresed on a 1.5% agarose gel.

Cytokine release from phagocytes *in vitro*. Blood neutrophils and peritoneal macrophages were incubated with LPS (100 ng/ml) for 3 h and 6 h, respectively, in the presence or absence of various concentrations of 18:0 LPC. In some experiments, blood neutrophils were preincubated with either G2A-specific antibody (1 μg/ml) or normal goat IgG (1 μg/ml) for 30 min before the addition of 30 μM 18:0 LPC to the medium. LPS (100 ng/ml) was added to the cells 30 min later, and TNF-α in the medium was measured 3 h after the addition of LPS.

Statistical analysis. Survival data were analyzed by the log-rank test. All other data were evaluated by ANOVA. The Bonferroni test was used for *post hoc* comparisons. $P < 0.05$ was considered to indicate statistical significance.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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1. Hoyert, D.L., Kochanek, K.D. & Murphy, S.L. Deaths: final data for 1997. *Natl. Vital Stat. Rep.* **47**, 1–104 (1999).
2. Huber-Lang, M.S. *et al.* Complement-induced impairment of innate immunity during sepsis. *J. Immunol.* **169**, 3223–3231 (2002).
3. Czermak, B.J. *et al.* Protective effects of C5a blockade in sepsis. *Nat. Med.* **5**, 788–792 (1999).
4. Docke, W.D. *et al.* Monocyte deactivation in septic patients: restoration by IFN-γ treatment. *Nat. Med.* **3**, 678–681 (1997).
5. Wang, S.D., Huang, K.J., Lin, Y.S. & Lei, H.Y. Sepsis-induced apoptosis of the thymocytes in mice. *J. Immunol.* **152**, 5014–5021 (1994).
6. Ayala, A. *et al.* Increased mucosal B-lymphocyte apoptosis during polymicrobial sepsis is a Fas ligand but not an endotoxin-mediated process. *Blood* **91**, 1362–1372 (1998).
7. Hotchkiss, R.S. *et al.* Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans. *J. Immunol.* **166**, 6952–6963 (2001).
8. Hotchkiss, R.S. & Karl, I.E. The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* **348**, 138–150 (2003).
9. Bone, R.C. Sir Isaac Newton, sepsis, SIRS, and CARS. *Crit. Care Med.* **24**, 1125–1128 (1996).
10. Huber-Lang, M.S. *et al.* Protective effects of anti-C5a peptide antibodies in experimental sepsis. *FASEB J.* **15**, 568–570 (2001).
11. Hotchkiss, R.S. *et al.* Prevention of lymphocyte cell death in sepsis improves survival in mice. *Proc. Natl. Acad. Sci. USA* **96**, 14541–14546 (1999).
12. Kabarowski, J.H., Xu, Y. & Witte, O.N. Lysophosphatidylcholine as a ligand for immunoregulation. *Biochem. Pharmacol.* **64**, 161–167 (2002).
13. Quinn, M.T., Parthasarathy, S. & Steinberg, D. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc. Natl. Acad. Sci. USA* **85**, 2805–2809 (1988).
14. Nakano, T., Raines, E.W., Abraham, J.A., Klagsbrun, M. & Ross, R. Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. *Proc. Natl. Acad. Sci. USA* **91**, 1069–1073 (1994).
15. Liu-Wu, Y., Hurt-Camejo, E. & Wiklund, O. Lysophosphatidylcholine induces the production of IL-1β by human monocytes. *Atherosclerosis* **137**, 351–357 (1998).
16. Coutant, F. *et al.* Mature dendritic cell generation promoted by lysophosphatidylcholine. *J. Immunol.* **169**, 1688–1695 (2002).
17. Sakai, M. *et al.* Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. *J. Biol. Chem.* **269**, 31430–31435 (1994).
18. Gomez-Munoz, A., O'Brien, L., Hundal, R. & Steinbrecher, U.P. Lysophosphatidylcholine stimulates phospholipase D activity in mouse peritoneal macrophages. *J. Lipid Res.* **40**, 988–993 (1999).
19. De Vries, H.E. *et al.* Acute effects of oxidized low density lipoprotein on metabolic responses in macrophages. *FASEB J.* **12**, 111–118 (1998).
20. Ngwenya, B.Z. & Yamamoto, N. Effects of inflammation products on immune systems. Lysophosphatidylcholine stimulates macrophages. *Cancer Immunol. Immunother.* **21**, 174–182 (1986).
21. Ramos, M.A. *et al.* Induction of macrophage VEGF in response to oxidized LDL and VEGF accumulation in human atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* **18**, 1188–1196 (1998).
22. McMurray, H.F., Parthasarathy, S. & Steinberg, D. Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. *J. Clin. Invest.* **92**, 1004–1008 (1993).
23. Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. Lysophosphatidylcholine as a possible second messenger synergistic to diacylglycerol and calcium ion for T-lymphocyte activation. *Biochem. Biophys. Res. Commun.* **178**, 1378–1385 (1991).
24. Nishi, E. *et al.* Lysophosphatidylcholine increases expression of heparin-binding epidermal growth factor-like growth factor in human T lymphocytes. *Circ. Res.* **80**, 638–644 (1997).
25. Nishi, E. *et al.* Lysophosphatidylcholine enhances cytokine-induced interferon gamma expression in human T lymphocytes. *Circ. Res.* **83**, 508–515 (1998).
26. Asaoka, Y., Oka, M., Yoshida, K., Sasaki, Y. & Nishizuka, Y. Role of lysophosphatidylcholine in T-lymphocyte activation: involvement of phospholipase A2 in signal transduction through protein kinase C. *Proc. Natl. Acad. Sci. USA* **89**, 6447–6451 (1992).
27. Savage, J.E., Theron, A.J. & Anderson, R. Activation of neutrophil membrane-associated oxidative metabolism by ultraviolet radiation. *J. Invest. Dermatol.* **101**, 532–536 (1993).

28. Nishioka, H., Horiuchi, H., Arai, H. & Kita, T. Lysophosphatidylcholine generates superoxide anions through activation of phosphatidylinositol 3-kinase in human neutrophils. *FEBS Lett.* **441**, 63–66 (1998).
29. Silliman, C.C. *et al.* Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium. *J. Leukoc. Biol.* **73**, 511–524 (2003).
30. Kabarowski, J.H., Zhu, K., Le, L.Q., Witte, O.N. & Xu, Y. Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. *Science* **293**, 702–705 (2001).
31. Muller Kobold, A.C. *et al.* Leukocyte activation in sepsis; correlations with disease state and mortality. *Intensive Care Med.* **26**, 883–892 (2000).
32. Solomkin, J.S., Jenkins, M.K., Nelson, R.D., Chenoweth, D. & Simmons, R.L. Neutrophil dysfunction in sepsis. II. Evidence for the role of complement activation products in cellular deactivation. *Surgery* **90**, 319–327 (1981).
33. Zhu, K. *et al.* Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.* **276**, 41325–41335 (2001).
34. Okajima, F. *et al.* Stimulatory and inhibitory actions of lysophosphatidylcholine, depending on its fatty acid residue, on the phospholipase C/Ca^{2+} system in HL-60 leukaemia cells. *Biochem. J.* **336**, 491–500 (1998).
35. Zantl, N. *et al.* Essential role of gamma interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis. *Infect. Immun.* **66**, 2300–2309 (1998).
36. Tzianabos, A.O. *et al.* IL-2 mediates protection against abscess formation in an experimental model of sepsis. *J. Immunol.* **163**, 893–897 (1999).
37. Weighardt, H. *et al.* Impaired monocyte IL-12 production before surgery as a predictive factor for the lethal outcome of postoperative sepsis. *Ann. Surg.* **235**, 560–567 (2002).
38. Nakahata, E., Shindoh, Y., Takayama, T. & Shindoh, C. Interleukin-12 prevents diaphragm muscle deterioration in a septic animal model. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **130**, 653–663 (2001).
39. Remick, D.G. *et al.* Combination immunotherapy with soluble tumor necrosis factor receptors plus interleukin 1 receptor antagonist decreases sepsis mortality. *Crit. Care Med.* **29**, 473–481 (2001).
40. Matsumoto, T. *et al.* Contribution of neutrophils to lipopolysaccharide-induced tumor necrosis factor production and mortality in a carrageenan-pretreated mouse model. *FEMS Immunol. Med. Microbiol.* **17**, 171–178 (1997).
41. Drobnik, W. *et al.* Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *J. Lipid Res.* **44**, 754–761 (2003).
42. Macphee, C.H. Lipoprotein-associated phospholipase A2: a potential new risk factor for coronary artery disease and a therapeutic target. *Curr. Opin. Pharmacol.* **1**, 121–125 (2001).
43. Corr, P.B. *et al.* Pathophysiological concentrations of lysophosphatides and the slow response. *Am. J. Physiol.* **243**, H187–H195 (1982).
44. Kishimoto, T., Soda, Y., Matsuyama, Y. & Mizuno, K. An enzymatic assay for lysophosphatidylcholine concentration in human serum and plasma. *Clin. Biochem.* **35**, 411–416 (2002).
45. Szucs, S., Varga, C., Ember, I. & Kertai, P. The separation of the granulocytes from different rat strains. A comparative study. *J. Immunol. Methods* **167**, 245–251 (1994).
46. Qureshi, M.A. & Dietert, R.R. Bacterial uptake and killing by macrophages. in *Methods in Immunotoxicology* Vol. 2 (eds. Burleson, G.R., Dean, J.H. & Munson, A.E.) 119–131 (Wiley-Liss, New York, 1995).
47. Weng, Z. *et al.* A DNA damage and stress inducible G protein-coupled receptor blocks cells in G2/M. *Proc. Natl. Acad. Sci. USA* **95**, 12334–12339 (1998).